Published in final edited form as:

J Microsc. 2014 January ; 253(1): 31–41. doi:10.1111/jmi.12095.

Atomic force microscopic investigation of respiratory syncytial virus infection in HEp-2 cells

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Summary

Respiratory syncytial virus (RSV) primarily causes bronchiolitis and pneumonia in infants. In spite of intense research, no safe and effective vaccine has been developed yet. For understanding its pathogenesis and development of anti-RSV drugs/therapeutics, it is indispensable to study the RSV–host interaction. Although, there are limited studies using electron microscopy to elucidate the infection process of RSV, to our knowledge, no study has reported the morphological impact of RSV infection using atomic force microscopy. We report the cytoplasmic and nuclear changes in human epidermoid cell line type 2 using atomic force microscopy. Human epidermoid cell line type 2 cells, grown on cover slips, were infected with RSV and fixed after various time periods, processed and observed for morphological changes using atomic force microscopy. RSV infected cells showed loss of membrane integrity, with degeneration in the cellular content and cytoskeleton. Nuclear membrane was disintegrated and nuclear volume was decreased. The chromatin of the RSV infected cells was condensed, progressing towards degeneration via pyknosis and apoptosis. Membrane protrusions of ~150–200 nm diameter were observed on RSV infected cells after 6 h, suggestive of prospective RSV budding sites. To our knowledge, this is the first study of RSV infection process using atomic force microscopy. Such morphological studies could help explore viral infection process aiding the development of anti-RSV therapies.

Keywords

Apoptosis; atomic force microscopy; pyknosis; respiratory syncytial virus

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Conflicts of interests

The authors declare no conflict of interest.

Introduction

Human respiratory syncytial virus belongs to the *Pneumovirus* class of *Paramyxoviridae* family and is one of the leading causes of bronchiolitis and pneumonia in infants (Collins, 1999; Hacking & Hull, 2002). RSV has a single-strand negative-sense RNA genome consisting of 10 genes encoding for 11 proteins, of which the attachment glycoprotein (G) and fusion protein (F) are crucial for viral attachment to the host and its entry into the host cell. Although G protein is responsible for the attachment, the F protein is essential for the fusion, budding and release of the virus (Ogra, 2004; Costello *et al.*, 2012). Once RSV is attached to the host cell, it fuses with the host cell membrane through its F protein. Although the mechanism of RSV infection is not clearly understood, based on the most common mechanism, nucleocapsid enters the host cell through F protein via clathrin-mediated endocytosis (Collins & Graham, 2008). This is followed by entry of viral RNA into host cells, replication and genome expression; these events are limited to the cytoplasm of the host and cytoplasmic organelles are not involved (Collins, 1999; Hacking & Hull, 2002). As the infection proceeds, the cells start losing their characteristic shape to enable syncytia formation. The virions acquire a lipid envelope and exude from the host cell through budding (Collins & Graham, 2008). The RSV pathogenesis escalates because of the destruction of the epithelial cells by virus replication resulting in edema, mucus secretion, influx of immune cells such as lymphocytes and macrophages.

It has been widely known that the pathogenesis of RSV is related to the host cell and its immune response (González *et al.*, 2012). Significant progress has been made in the understanding of RSV infection, its pathogenesis, host cell interaction, molecular signalling involved in programmed cell death and adaptive and innate immunity (Oshansky *et al.*, 2009). Several cellular models have been used to understand the underlying mechanisms of RSV infection, viral culture and propagation including human epidermoid cell line type 2 (HEp-2) and the African green monkey kidney cell line, Vero (Collins, 1999). Apart from molecular and immunological perspective, several microscopic studies have also been conducted using scanning and transmission electron microscopy (EM), revealing the structure and intracellular details of an RSV infected cell.

EM is an excellent imaging method to study virion structure in detail (Norrby *et al.*, 1970; Bachi & Howe, 1973). EM has been used to study the arrangement of various proteins in RSV such as G, GM1 and Rho A, their role in the RSV infection (Jeffree *et al.*, 2003; Gower *et al.*, 2005) and maturation of viral progeny at the polarized cells (Roberts *et al.*, 1995). Human RSV differs from that of bovine RSV in having the 'bridging effect' because of the aggregation of RSV particles while evading the host ovine kidney cells (Belanger *et al.*, 1988). Although EM provides great specifics about the viral structure, it is limited regarding the three dimensional analysis of the virus and/or the virus infected cell. Also, the sample preparation might have adverse effects on the real time analysis of the virus and its host (Bachi & Howe, 1973; Chen, 2007). Therefore, to obviate this problem, researchers have utilized atomic force microscopy (AFM) in the study of host-pathogen interactions as well as in the analysis of the pathogen itself.

Several applications of AFM have been reported in the fields of biology and biomedicine (Chang *et al.*, 2012). Recently, AFM has been used for studying the physicochemical properties of intact and disrupted virions (Liu *et al.*, 2012; Martinez-Martin *et al.*, 2012; Cartagena *et al.*, 2013). AFM has been previously reported to be useful in the contact mode to image living mammalian cells in culture to visualize the cell membrane, cytoskeleton and their conformational changes because of cell division, cell locomotion and transformation by viruses (Kuznetsov *et al.*, 1997). Recently AFM was used to study the antiviral activity of pinostrobin against herpes simplex virus-1 using AFM with significant changes in the morphology and size of herpes simplex virus (Wu *et al.*, 2011). AFM revealed changes in the topography and phase images with increasing time, including shedding of the resulting in the inactivation of virus. Similarly, the structure and intracellular details of the mimivirus have been revealed through AFM upon serial degradation with lysozyme and bromelain (Kuznetsov *et al.*, 2010). AFM is applicable to study chemically induced morphological changes at the nanometre level. AFM has also been used for accurate determination of the size and shape of virus particles, the architecture and mechanical properties of their surfaces including elasticity or brittleness, because viruses are subjected to several internal and external forces (Kuznetsov *et al.*, 2001; Chen, 2007; Dubrovin *et al.*, 2007; Mateu, 2012). We have previously reported the use of AFM for studying the adhesion and mechanical properties of RSV infected HEp-2 cells (Pfendt *et al.*, 2011). Viruses use these mechanical forces to resist the host defence and to infect other cells. The AFM has proved to be highly proficient in such studies, helping in the understanding of various molecular determinants of these viruses. Specifically, AFM was employed in a study to image HIV and HIV infected lymphocytes to compare the effects of different proteases on the morphology of the virus and the infected host cells (Kuznetsov *et al.*, 2003). Although AFM is not a penetrating technique like EM, it can be employed to investigate intracellular entities of a virus. In fact, AFM was exploited to study the intracellular details of viruses (Malkin *et al.*, 2003; Kuznetsov *et al.*, 2005) upon treatment with detergents and reducing agents. A study comparing both TEM and AFM analyses of the recombinant Adeno-associated virus showed that AFM has a pronounced role while examining several viral particles as the size and shape could be quite different from the TEM images. Hence, AFM could play a significant role in understanding a different aspect of virion morphology because it can be used to study cells and virus particles under their natural and physiological conditions.

In this study, HEp-2 cells infected with RSV as a model using AFM to investigate the morphological and intracellular changes in the host cells. We evaluated the consequences of RSV infection over time on HEp-2 cell morphology as well as cytoplasmic and nuclear content. We also determined the applicability of AFM in various modes for an in-depth evaluation of outcome of RSV pathogenesis, from the cell's perspective. We observed that during the time course of RSV infection, cells suffer critical changes. This understanding would help in the development of antivirals or vaccine against RSV early in the infection process. In addition, this work demonstrates that AFM holds significant promise as a tool to visualize the three dimensional changes occurring during RSV infection *in vitro*.

Materials and methods

Cell culture and RSV stock preparation

HEp-2 were purchased from American type collection culture (ATCC, Manassas, VA, U.S.A.) and were cultured in Minimal essential medium (MEM) (Life Technologies™, Carlsbad, CA, U.S.A.) supplemented with 75 U/mL Penicillin, 100 mg/mL kanamycin and 75 mg/mL streptomycin and either 2% or 10% foetal bovine serum. RSV Long strain was obtained from the ATCC (Manassas, VA, U.S.A.) and stocks were prepared as described previously (Sun *et al.*, 2008; Vig *et al.*, 2009).

RSV infection

HEp-2 cells were seeded on silica coated cover slips (20 mm in diameter) in 12 well tissue culture dishes at 1.8×10^5 cells per well overnight in incubator at 37°C supplemented with 5% CO₂. After the cells reached 60% confluency, they were infected with 100 PFUs of RSV. The infection was carried out for various periods from 30 min to 6 h. This was followed by fixing the cells with 200 μ L methanol for 30 min, 200 μ L distilled water for 15 min and washed successively with 70%, 90% and absolute alcohol for 5 min each. Following fixation and dehydration, cover slips containing HEp-2 cells were analysed using AFM as described below.

AFM analysis

AFM analysis was performed using the XE-100 Atomic Force Microscope (Park Systems, Santa Clara, CA, U.S.A.) at the University of Louisville (Louisville, KY, U.S.A.). RSV infected HEp-2 cells were fixed in methanol, placed onto a slide, dried and visualized under the microscope. Non-contact mode and standard silicon cantilevers (Pacific Nanotechnology, Santa Clara, CA, U.S.A.) 450 μ m in length and 20 μ m in width were employed for imaging. The cantilever oscillation frequency was tuned to the resonance frequency of approximately 256 kHz. The set point voltage was adjusted for optimum image quality. Height, phase and amplitude information were recorded at a scan rate of 1Hz, and in 512 $\times$ 512 pixel format. AFM images in a large scanning area were processed using NanoRule software (Pacific Nanotechnology).

Imaging of cells began by identifying large areas (greater than 250 $\times$ 250 μ m) on the cover slips where cells have remained attached throughout the fixing and dehydration process. Although areas of high cell density were imaged, the focus for this work was on areas of low cell density because it is in these areas that single cells can be found which allowed for ease of focused imaging and image analysis. Most of these images are taken on the scale of 50 $\times$ 50 μ m to be able to image an entire cell. Several individual cells from each cover slip were imaged leading to at least a dozen cells imaged at each time step with many more imaged during the search for these cells.

Results

Morphological and nuclear changes attributed to RSV infection

During infection of HEp-2 cells with RSV, the cells underwent several nuclear and morphological changes. These changes included membrane fusion of the adjacent infected cells. The un-infected cells showed intact cellular and nuclear membranes, with healthy and voluminous nucleus, with an elliptical shape characteristic of the HEp-2 cells (Fig. 1). The nucleus was intact in shape and with a step height of 1.2 μm . The uninfected cells showed normal integrity of the cytoplasmic content with no deformation of the cell surface. There was no damage observed in either the membrane or nucleus of the uninfected cell.

Morphological changes in the cell membrane upon RSV infection

We observed that HEp-2 cells infected with RSV start losing their typical ellipsoid shape with increasing incubation time. A short duration of infection with RSV (30 min) resulted in the elongation of the cells as compared to normal cells (Fig. 2A) and the appearance of depressions in the cellular membrane (Fig. 2B). These depressions are indicative of entry of RSV into cells within 30 min. of infection. The cell membrane in case of the uninfected cells was normal in appearance (i.e. no dip or elongation was observed). The elongation of cell also led to the morphological changes of the nucleus that are significantly different in RSV infected cells in comparison to uninfected cells (Fig. 2A).

We also observed that RSV infection caused decrease in the step height of the membranes after the cells were infected with RSV for 30 min when compared with the cell without RSV infection (Figs. 3A–C). The overall step height of the cell (defined as the distance between the surface of the cover slip and features on the cell) shown in the line scan of the image, is directly related to the volume of the cell such that diminutions in the step height are ultimately caused by shrinkage in the cell volume. The overall step height continued to decrease with increasing infection times up to 3 h (Fig. 3C). The reduction in volume and the step height of the cell was correlated with increasing exposure times (Figs. 3B, C). The step height for uninfected cells ranged between 25 and 400 nm (Fig. 3D), whereas for cells infected with RSV over 30 min it was reduced to ~ 150 nm (Fig. 3E Box). After 3 h of RSV infection, there were multiple peaks ranging from approximately 10–100 nm, indicating that the nucleus is disintegrated because of pyknosis (Fig. 3F). Although there is a great deal of variability in the step height not only between cells, but also within individual cells, the overall trend of decreasing cell height, and hence cell volume, holds as infection time increases. These results indicate and confirm that cellular content is being lost and damaged as the RSV infection continues.

The changes in the cell membrane became more profound and prominent with increasing infection time. RSV infection progresses with time which is marked by the beginning of syncytia formation approximately after 2.5 h of RSV infection where the ridges made by RSV became more evident in comparison to the normal cell (Figs. 4A, B). As seen in Fig. 4(B), a cell adjacent to an RSV infected cell, progressing towards syncytial formation, thus further spreading the infection. The indentation made by RSV infection become numerous in cells infected for 2.5, 3 and 5 h (Figs. 4B–D). Also, the surface of the RSV infected cells

was found to have a coarse morphology as opposed to the control (uninfected) cell which had comparatively smoother surface. Numerous depressions were observed in cells infected with RSV for 3 h, a clear evidence of increase in RSV growth and damage to the cell membranes (Fig. 4C). As the RSV infection continues to 5 h, cell membranes are almost completely damaged and very little membrane structure is seen (Fig. 4D). In addition, some protuberances appear on cell membrane after 5 h of infection which correspond to pronounced cell damage. The protuberances observed in our AFM studies could be correlated to the budding of progeny viruses (Fig. 4D). These results are similar to those obtained in our earlier work in which changes in the chemical moiety exposed on the cell surface changed as a function of infection time (Pfundt *et al*, 2011). A statistical analysis performed in that work showed that as infection time increased, the hydrophobic nature of the cell decreased, as measured by the adhesion between the cell and a hydrophilic tip, which corresponded to a destruction of the cell membrane.

After 6 h of RSV infection, the cell encountered a severe damage pertaining to its three dimensional shape, organization of the intracellular content and membrane integrity. The cell's cytoskeletal volume began to reduce with loss in its apical ends finally culminating in the shortening of the cellular appendages (Fig. 5). Amplitude imaging was utilized in this case as it is more sensitive to small but distinct changes in height that might be missed in a typical topography image which helps in determining the presence and position of the cellular and nuclear membranes. At this juncture (Fig. 5B), none of the cellular boundaries are distinct (as they are in Fig. 5A) which is an indication of cellular destruction. The AFM images thus demonstrate cell damage, which occurs in the later stage of RSV infection, in this case after 6 h.

Nuclear changes during RSV infection

Although, RSV replication and maturation occur in the cytoplasm, the concurrent cytoplasmic activities of RSV increase the cytoplasmic burden leading to an increase in the perinuclear content, compressing against the nucleus. We found that HEp-2 cells (Fig. 6A) after infection with RSV after 30 min did not show noticeable changes in the nuclear membrane, size and volume as the RSV attaches and enters the host cells but has yet to reach the threshold of the infection (Fig. 6B). However, as the infection progresses, the nucleus tends to undergo considerable changes in its shape and volume after 6 h of RSV infection (Fig. 6C). Although, nuclear content is seen, there is no clear nuclear membrane. The height to volume ratio of the nucleus tends to decrease significantly than the uninfected cell.

There was a considerable reduction in nuclear volume as compared to the control cell when cells were infected for 4 and 6 h (Figs. 7A–C). The nuclear content is significantly reduced and nuclear fragmentation is evident after cells are infected for 4 h (Fig. 7B and corresponding line image in Fig. 7E) as evidenced by the lack of the ridge that corresponds to the presence of the nuclear membrane (highlighted by the box in Fig. 7D). The step height of the nucleus in uninfected cell was measured by a single peak $\sim 1 \mu\text{m}$ (Fig. 7D), whereas the nuclear step height of the cells infected for 4 h showed two peaks around $\sim 1 \mu\text{m}$, indicating the disintegration of the nucleus. Although, the line graph in Fig. 7(E) depicts

only two peaks, the corresponding image in Fig. 7(B) clearly shows many more fragments of the chromatin material. The nuclear disintegration is further enhanced and after 6 h of infection, very little nuclear content is observed (Figs. 7C, F). This is more evident from the size of the nuclear step height peaks, which are ~20 nm single peak in uninfected cell, two peaks ~5 nm after 4 h of infection and in cells with RSV infection for 6 h, a single peak of ~5 nm. The increased cytoplasmic activities lead to a compression in the cytoplasm, pressing against the nucleus. This could also be attributed to the cytopathic effects of the host cells as a measure to prevent infection in the adjacent cells, driving the cells towards a pyknotic stage.

Budding of RSV

After establishing the process of attachment, entry and replication of the virus which were shown in RSV infection at different infection durations, we were interested in the process of RSV budding. It was very interesting to see the RSV budding out of the cell surface at 6 h of RSV infection. The cellular surface was imaged using phase imaging on the cells with or without RSV infection. Phase imaging allows for the visualization of changes in the physicochemical properties across a surface of interest. The cells infected with RSV for 6 h revealed several pleomorphic contusions on the cell surface which were absent on control cells (Figs. 8A, B). These prominent bulges were observed in cells with RSV infection in comparison to cells without infection. These bulges directly correlate with the budding of RSV. To confirm the RSV budding, we measured the sizes of these bulges which ranged between 200 and 300 nm in diameter (Fig. 8C). This size correlates with the known average diameter of RSV which is between 1200 and 300 nm. To further confirm these bulges, we measured the height (Figs. 8C, D), which showed significant difference in height compared to other surface area of cells without any bulges. Therefore, we confirm that these bulges are the result of RSV budding (Fig. 8).

Discussion

AFM has been widely used in the field of virology because of its ability to image the structure of biomolecules or biosurfaces with high resolution, without compromising the quality of images because of the physiological conditions of the sample (Santos & Castanho, 2004). It allows the visualization of *in situ*, real-time dynamics of various biological events and provides insight into different local chemical, physical and mechanical properties of a sample. We have shown for the first time, the morphological and nuclear changes with a clear evidence of pyknosis and apoptosis in RSV infected HEP-2 cells using AFM.

It is well known that RSV infection leads to critical changes in the host cells morphology. These changes are a result of the host cell response to the infection. In this study, we have shown that the HEP-2 cells undergo severe morphonuclear changes because of RSV infection. These changes are attributed to the interactions between cellular actin and viral components which stimulate a temporary elevation in the β -actin, resulting in the formation of characteristic cytoplasmic extensions which are known to contain the viral particles (Ulloa *et al.*, 1998). We have observed similar changes occurring on the cell membrane of RSV infected cells (Figs. 1–5). The cell membrane suffered critical distortions in its

cytoskeletal content such as the flattening of the apical ends and an overall decrease in the step height of the cell. These cytoplasmic extensions are thought to be used by RSV for its motility through the cytoplasm and across the neighbouring cells (Ulloa *et al.*, 1998).

Similarly, most of the paramyxoviruses exploit cytoskeletal components to complete their life cycle, such as the micro-tubules (e.g. Measles and Sendai virus) or actin (e.g. RSV and PIV3). Any changes in the microtubule or actin function could interfere in various steps of the life cycle of RSV including entry into the host cell, formation of cell-associated virus, virus escape, exacerbation of the infection and syncytium formation (Kallewaard *et al.*, 2005). In our study, we have elucidated that the cell membranes of the RSV infected cells lost their characteristic shape and the cytoskeleton was reduced and elongated. The loss in the normal shape and cytoskeleton elevated with increasing infection times with RSV. These data are substantiated with the microarray studies suggesting changes in the expression levels of genes associated with the organization of cytoskeleton, inflammation inducing genes, cytokines, chemokines and those involved in cell growth/proliferation upon RSV infection (Fjaerli *et al.*, 2007; Huang *et al.*, 2008; Smith *et al.*, 2012). The RSV infected human bronchial epithelial cell line (BEAS-2B, subclone S6) showed a down regulation in the gene TUBB1 (tubulin1-needed for microtubule organization) and upregulation of TACR1 (Receptor for tachykinin substance P) which is associated with RSV-mediated airway inflammation and dysfunction (Huang *et al.*, 2008). Similarly, in a study involving mRNA microarray data sets changes in the gene, TUBB1 were observed (Smith *et al.*, 2012). RSV infection down regulated the genes and a pathway involved in the regulation of actin cytoskeleton (Fjaerli *et al.*, 2007).

As opposed to Kallewaard *et al.*, (Kallewaard *et al.*, 2005), we have successfully utilized AFM to observe the surface of RSV infected cells (Figs. 2–5) which demonstrated the loss of apical cytoskeleton, whereas no distortion in the cell morphology of the uninfected cells was observed (Fig. 1). RSV fusion protein has a role in the fusion of RSV to host cells and its internalization, leading to loss of the cilia in the infected cells, which could help RSV evade from epithelial clearance and infect adjacent cells (Eckardt-Michel *et al.*, 2008).

We observed critical changes in the nucleus of RSV infected cells concerning the structure and size of the nucleus (Figs. 6 and 7). The reduction in the nuclear volume can be attributed to the fact that RSV infection reaches a noticeable level in terms of protein expression and genome replication around 6 h. Our findings evidently contradict a previous report suggesting no changes in the nucleus of Vero cells infected with RSV (Norrby *et al.*, 1970). We found that the nuclear content and volume, as measured by the changes in the step height of the cells, reduced remarkably (Fig. 7) which tends to be because of the pyknosis in the nucleus to prevent the infection from proceeding to the neighbouring cells. It is imperative for the infected cells to undergo ‘self-destruction’ through the manifestation of cytopathic effects including pyknosis leading to apoptosis and necrosis rather than supporting the virus for further infection. Also, apoptosis enhances antigen presentation thereby stimulating the host immune system. It has been reported that RSV infection could lead to an increase in the transcription of both pro- and antiapoptotic factors especially the tumour necrosis factor-related apoptosis-inducing ligand and its functional receptors death receptor 4 (DR4) and DR5 (Kotelkin *et al.*, 2003). RSV infection also caused upregulation

of several pro-apoptotic factors belonging to the Bcl-2 family and caspases 3, 6, 7, 8, 9 and 10, which represent the death receptor- as well as mitochondrion-dependent apoptotic pathways. RSV F protein has also been associated with the manifestation of p53-dependent apoptosis, triggered through caspase-dependent cell death, causing the ephemeral loss of membrane integrity (Eckardt-Michel *et al.*, 2008). It has also been shown that 4 h after RSV infection in BEAS-2B cell line, the pro-apoptotic gene caspase 8 and Fas associated death domain are down regulated suggesting the apoptotic suppressor effect of RSV, whereas the gene BBC3 (bcl-2 binding component 3) is upregulated which is an upregulated modulator of apoptosis suggesting the host cells commitment to programmed cell death (Huang *et al.*, 2008). However, it has been shown that early apoptosis might be unfavorable for virus replication; it is a defensive measure of the host to prevent further infection at an early stage. Thus; it becomes imperative for RSV to halt apoptosis long enough to help the development of the polykaryons which ultimately lyse to release mature RSV (Kotelkin *et al.*, 2003). Moreover, HEp-2 cells persistently infected with RSV have been shown to develop resistance to apoptosis to recover from the infection as compared to cells which underwent lytic cycle (Martínez *et al.*, 2009). Also, RSV has been found to infect only the cells which are committed to a stress pathway and the RSV infected cells showed an increased number of stress granules which was found to enhance RSV infection (Lindquist *et al.*, 2010). This further substantiates our hypotheses that the RSV infection leads to the distortion in the cytoplasm of the infected cell.

We observed the budding RSV from surface of the HEp-2 cells after 6 h of infection (Fig. 8). It is known that during the first 6–8 hours of infection RSV accumulates F protein in the host cells that helps it spread infection to neighbouring cells, which promotes the arousal of these protrusions from the infected cells. In an earlier report, RSV infected Vero cells have been shown to possess several such projections corresponding to budding RSV which were often clustered (Norrby *et al.*, 1970). Similar results were obtained in a study on HIV and HIV infected lymphocytes where the infected cells were shown to have accumulated the budding virions (Kuznetsov *et al.*, 2003). Several ‘tufts’ of presumably gp120 (an envelope protein of HIV) were found on the cell surface. Similarly, RSV F and G proteins have been shown to accumulate on the surface of infected cells (Low *et al.*, 2008), henceforth, supporting our assumption of RSV-F agglomeration on the cell surface helping the budding of RSV from the host cell membrane (Fig. 8). Also, when NIH 3T3 cells were infected in culture with mouse leukemia virus (MuLV), an oncogenic retrovirus, and studied using AFM, the budding virions were seen emerging from the cytoskeletal matrix (Kuznetsov *et al.*, 2002). They found that the cells were covered up with tube-like structures arising from the failure of the virions to complete morphogenesis and budding successfully from the surface of host cells. These findings closely resemble ours, where we observed several protrusions arising from the infected cells were between 150 and 200 nm in diameter, matching the average size of RSV.

So far several studies have been conducted using various microscopy techniques to gain an insight into the details of RSV infection. But, to our knowledge none of the studies have reported the structural damage occurring in the host cells using AFM. AFM is a great tool to study biological processes and phenomenon and it has been extensively used in various

aspects of virology. We further support the utilization of AFM to study the process of viral infections to decipher important steps and to understand the host-cell interactions.

Conclusion

To our knowledge, this is the first study showing significant mutilation to the cell membrane and nucleus of an RSV infected HEp-2 cell using AFM. This study highlights that RSV infection causes severe distortions in the cytoskeleton of host cell membrane and nucleus. The nuclear changes clearly demonstrate the loss of the chromatin showing the occurrence of pyknosis which destines the cell for apoptosis. This provides means to study the three dimensional aspect of not only the surface of an object of interest but also to a great extent its inner content. Although, the importance of EM in the study of viruses cannot be overshadowed by AFM, we do suggest that EM studies should be supplemented with AFM studies to gain comprehensive cellular structural changes attributed because of viral infections.

Acknowledgments

The authors acknowledge Dr. Lekha Adiani for assistance in cell culture and RSV infections, Kyoung G. Lee and Adam Pfendt (University of Louisville) for assistance with AFM imaging. Financial support from NSF-CREST (HRD-1241701) and NSF-HBCU-UP (HRD-1135863) is acknowledged.

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Fig. 1.

AFM analysis of an uninfected HEp-2 cell: (A) HEp-2 cell ($50 \times 50 \mu\text{m}$) with normal cellular and nuclear shape as well as content and (B) inset shows the nucleus with intact morphology and no visible signs of aberration. The uninfected cells showed intact cell membrane and voluminous nucleus with no evidence of pyknosis or chromatin degradation

Fig. 2.

Effect of RSV attachment and infection on HEp-2 cell membrane: (A) Uninfected cell ($50 \times 50 \mu\text{m}$) with smoother cell membrane and (B) HEp-2 cell ($70 \times 70 \mu\text{m}$) after 30 min of RSV infection. The cell suffers severe changes in the cell morphology because of the attachment and internalization of RSV into the host cell. The boxes in (B) highlight the depressions in the cell membrane suggestive of possible sites of RSV attachment and internalization.

Fig. 3.

Effect of RSV infection on the cell membrane and nucleus: (A) Noninfected HEp-2 cell, (B) HEp-2 cells infected with RSV for 30 min and (C) HEp-2 cells infected for 3 h with RSV. The nuclear content condensed gradually with time after 30 min of RSV infection (B) as compared to the uninfected HEp-2 cells (A), the size and the volume of the nucleus is reduced with condensation as can be seen in the decrease of the average cell height as visualized in the corresponding line scans in (E) and (D). After 3 h of RSV infection the impairments in the nucleus become more prominent (C) as the chromatin material loses its intactness and appears to be fragmented suggesting the occurrence of pyknosis. Also, the cell membrane shows a continued decline in the step height (F) suggestive of the deterioration in cytoplasmic content.

Fig. 4.

Cell membrane changes during RSV infection: (A) A noninfected HEp-2 cell ($50 \times 50 \mu\text{m}$), (B) HEp-2 cell after 2.5 h after RSV infection ($70 \times 70 \mu\text{m}$), (C) HEp-2 cell after 3 h of RSV infection ($40 \times 40 \mu\text{m}$) and (D) HEp-2 cell 5 h after RSV infection ($50 \times 50 \mu\text{m}$). The rectangles indicate the depressions on the host cell membrane which become more prominent and numerous with increasing time. These depressions represent the sites of RSV entry and the rearrangements in the actin and microtubules and prospective cytopathic effects.

Fig. 5. Effect of RSV infection on the cytoskeleton Amplitude images of (A) a non-infected HEP-2 cell and (B) HEP-2 cell infected with RSV for 6 h. RSV infection affects the organization and appearance of the microtubules as evident in (B). The cell has more flattened surface and reduced cellular appendages.

Fig. 6. Nuclear changes in HEp-2 cells: (A) Noninfected HEp-2 cell ($50 \times 50 \mu\text{m}$), (B) HEp-2 cell infected with RSV for 30 min ($70 \times 70 \mu\text{m}$) and (C) HEp-2 cells infected with RSV for 6 h ($50 \times 50 \mu\text{m}$). The uninfected cell (A) shows a healthy and voluminous nucleus with large surface area. The nucleus start narrowing gradually (B) and the changes in the size and shape of the nucleus become more prominent at 6 h postinfection (C). Boxes are included to highlight the nuclear area of the cell in each image.

Fig. 7.

Effect of RSV infection on the host nucleus: (A) HEp-2 cell without RSV infection, (B) HEp-2 cell after 4 h of RSV infection and (C) HEp-2 cell 6 h post-RSV infection. Line scans in D through F correspond to the highlighted lines in A through C and show a more detailed view of the height variation across the diameter of the cell. The nucleus of the cell without RSV infection appears very large, voluminous and intact (A) as compared to the infected cells. After 4 h of infection, the nucleus starts disintegrating (B) with fragmentation of the chromatin. After 6 h of RSV infection, the nuclear volume is severely reduced (C), with meager nuclear content being visible.

Fig. 8.

Budding of RSV from HEp-2 cells: Phase images of (A) HEp-2 cell without RSV infection, (B) budding of RSV from the host cells infected with RSV for 6 h, (C) are topography image of HEp-2 cell 6 h after RSV infection highlighting distortion of the cell membrane by the budding RSV. (D) Three dimensional topographic view of (C). The cell membrane becomes rough with numerous protuberances appearing on its surface.